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INHIBITORY EFFECTS OF UBENIMEX (BESTATIN) ON THE INVASION OF UTERINE CERVICAL CARCINOMA CELLS AND THEIR PRODUCTION AND ACTIVATION OF GELATINASE A

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Key Words: Aminopeptidase, bestatin, gelatinase A, invasion, uterine cervical carcinoma.
Subjects: Uterine cancer cells.
Abbreviations: Ala-MCA = alanine-4-methylcoumarin-7-amide, AMC = 7-amino-4-methylcoumarin, Arg-MCA = arginine-4-methyl-coumarin-7-amide, ECM = extracellular matrices, MCA = 4-methylcoumarin-7-amide, MMPs = matrix metalloproteinases, MTT = 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, PBS = phosphate-buffered saline, SDS = sodium dodecyl sulfate, TCM = tumor conditioned medium.

Abstract

The present study was undertaken to investigate the effects of the aminopeptidase inhibitor ubenimex (bestatin) on the invasive activity of cultured human uterine cervical carcinoma cells. The invasion of squamous cell carcinoma OMC-1 and SKG-IIIb cells, and adeno-

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carcinoma OMC-4 and CAC-1 cells into reconstituted basement membrane (Matrigel) was inhibited by the presence of bestatin in a concentration-dependent manner. However, bestatin did not have any effect on tumor cell proliferation or migration. Immunoblot analysis of tumor-conditioned medium showed that the treatment of tumor cells with bestatin resulted in the reduction of the 72 kDa gelatinase level (gelatinase A, latent form) in the four cell lines examined, and the reduction of the 68 kDa gelatinase level (gelatinase A, active form) in SKG-IIIb cells. Bestatin inhibited hydrolyzing activities towards substrates of aminopeptidases in tumor cells, but did not directly inhibit gelatinase A. These results suggest that bestatin may inhibit the invasion of uterine cervical carcinoma cells possibly through the inhibitory mechanisms for production and activation of gelatinase A modulated by tumor aminopeptidases.

Introduction

In the process of metastasis formation, tumor cells must pass through basement membranes and extracellular matrices (ECM), which consist of adhesive molecules such as fibronectin, laminin, collagen, and other glycoproteins and proteoglycans (Fidler, Gersten *et al.*, 1978; Liotta, Rao *et al.*, 1983). Tumor cell invasion of ECM is a complex process involving cell attachment, migration, and the degradation of tissue barriers caused by various proteolytic enzymes secreted by tumor cells. Matrix metalloproteinases (MMPs) are members of proteolytic enzymes which can degrade native collagens and other ECM components (Woessner, 1991). Since their substrates are the major components of ECM, the increased expression of MMPs by malignant tumor cells is believed to play an essential role in invasion and metastasis (Liotta, Steeg *et al.*, 1991; Matrisian, 1990).

Ubenimex, designated bestatin, is a dipeptide discoverd in the culture medium of *Streptomyces olivoreticuli* (Umezawa, Aoyagi *et al.*, 1976) and is clinically used as a biological response modifier (Ota and Ogawa, 1990). Bestatin is known to competitively inhibit the activity of aminopeptidases B and N on cell surfaces, to stimulate the release of various cytokines by immuno-competent cells, and modulate the function of anti-tumor effectors such as macrophages, NK cells, and cytotoxic T cells (Abe, Fujii *et al.*, 1990). In addition to these effects, bestatin has been shown to have inhibitory effects on tumor cell invasion

in vitro. Saiki, Murata *et al.* (1989) reported that bestatin inhibited the invasive ability of B16-BL6 melanoma cells and Lewis lung carcinoma (3LL) cells into reconstituted basement membrane (Matrigel)-coated filters through a mechanism involving its inhibitory action on aminopeptidases in these tumor cells. Yoneda, Saiki *et al.* (1992) also demonstrated that bestatin inhibited the invasion of human renal cell carcinoma SN12M cells into Matrigel and induced the reduction of active and latent forms of MMP-2 (gelatinase A) produced by SN12M cells. However, inhibitory effects of bestatin on the invasion of malignant tumor cells of gynecological origin has not been elucidated.

In the present study, we investigated the biological effects of bestatin on proliferation, migration and invasion of uterine cervical carcinoma cells together with the inhibitory effects of bestatin on gelatinase A secreted by these cells.

Materials and Methods

Cell Culture

Four human uterine cervical carcinoma cell lines were used. OMC-1 (Ueda, Ueki *et al.*, 1989) was established from a Virchow metastatic lesion of moderately-differentiated epidermoid carcinoma, and OMC-4 (Ueda, Ueki *et al.*, 1993) was established from a primary lesion of well-differentiated adenocarcinoma at our laboratory. SKG-IIIb (Nozawa, Udagawa *et al.*, 1983) originating from moderately-differentiated epidermoid carcinoma was kindly provided by Dr. S. Nozawa, Keio University, Tokyo, and CAC-1 (Koizumi, Uede *et al.*, 1988) originating from well-differentiated adenocaricnoma was kindly provided by Dr. O. Hayakawa, Sapporo Medical University, Sapporo. All cell lines were maintained as monolayer cultures in Ham's F-12 medium (Flow Laboratories Inc., Irvine, Scotland) supplemented with 10% fetal bovine serum (Mitsubishi Chemical Co., Tokyo) at 37°C in a humidified incubator with 5% CO_2 in air. These cells were grown to confluence in 75cm^2 tissue culture flasks (Nunc, Roskilde, Denmark), washed with phosphate-buffered saline (PBS), and then harvested after a brief treatment with 0.1% trypsin solution containing 0.02% EDTA (Flow Laboratories Inc.). Tumor conditioned medium (TCM) was prepared from the culture supernatant of the cells. Briefly, confluent monolayers of tumor cells grown in 6cm^2 plastic dishes (Corning 25010, Iwaki Glass, Tokyo) were rinsed twice with serum-free Ham's F-12

medium and incubated at 37°C for 48 hr with 4 mL of serum-free medium. The TCM samples thus obtained were derived from equal numbers of tumor cells, condensed 5-fold by using Centricon-10 microconcentrators (Amicon, Division of W.R. Grace & Co., Columbia, MA) and then stored at -80°C until use.

Chemical Reagents and Antibody

Ubenimex (bestatin) was prepared by Nippon Kayaku Co., Ltd., Tokyo. 3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was obtained from Wako Pure Chemical Industries, Ltd., Osaka. Dimethyl-sulfoxide was purchased from Sigma Chemical Co., St. Louis, MO. Purified human fibronectin was purchased from Iwaki Glass. Basement membrane Matrigel (containing laminin, collagen type IV, heparan sulfate proteoglycan and entactin) was obtained from Collaborative Research Inc., Bedford, MA. Alanin-4-methylcoumarin-7-amide (Ala-MCA) and arginine-4-methylcoumarin-7-amide (Arg-MCA) were purchased from Peptide Institute Inc., Osaka. Anti-human MMP-2 (gelatinase A) mouse monoclonal antibody was purchased from Fuji Pharmaceutical Industries Ltd., Toyama.

MTT Assay

Effects of bestatin on the proliferation of cultured uterine cervical carcinoma cells were examined by MTT assay with some modifications as previously described (Yamada, Ueda *et al*., 1991). 1×10^4 cells in a volume of 100 µL of growth medium per well were uniformly seeded into 96-well microplates (Nunc), and incubated at 37°C for 48 hr. Each well was washed twice with serum-free Ham's F-12 with 0.1% BSA (Sigma) and cultured for another 24 hr in the same medium. The medium was then removed and replaced with 100 µL of serum-free Ham's F-12 with 0.1% BSA containing various amounts of bestatin. After further incubation at 37°C for 24 hr, 50 µL of MTT dissolved in PBS at a concentration of 2 mg/mL was added to each well, and the plates were incubated at 37°C for 6 hr. The medium was then removed, 200 µL of dimethyl-sulfoxide was added to each well, and the plates were agitated for 5 min. The absorbance was then read at 570 nm in a scanning spectrophotometer (Hitachi Ltd., Tokyo). All experiments were performed in quadruplicate and repeated three times.

Haptotactic Migration Assay

Tumor cell migration along a gradient of substratum-bound fibronectin was assayed in Chemotaxicell culture chambers (Kurabo, Osaka) according to the methods reported by McCarthy and Furcht (1984) with some modifications. Polyvinylpyrroridone-free polycarbonate filters with 8.0 µm pore size were precoated with 10 µg of fibronectin in a volume of 50 µL of PBS on the lower surface, and dried overnight at room temperature under a hood. The coated filters were washed extensively in PBS and then dried immediately before use. Log-phase cell cultures of tumor cells were harvested with 0.1% trypsin containing 0.02% EDTA, washed twice with serum-free Ham's F-12, and resuspended to a final concentration of 3 x 10^6/mL in serum-free Ham's F-12 with 0.1% BSA. 200 µL of cell suspension with or without various amounts of bestatin was added to the upper compartment, and 600 µL of serum-free Ham's F-12 with 0.1% BSA was immediately added to the lower compartment. The chambers were then incubated for 24 hr at 37°C in a 5% CO_2 air. The filters were fixed with ethanol, and stained with hematoxylin. Cells on the upper surface of the filters were removed by wiping with a cotton swab. The cells that had migrated to various areas of the lower surface were manually counted under a microscope at a magnification of 400. Each assay was performed in triplicate.

Invasion Assay

The invasive activity of tumor cells was assayed according to the method reported by Albini, Iwamoto *et al.* (1987) with some modifications. Briefly, the lower surface of the filters was precoated with fibronectin as described above. The Matrigel diluted to 500 µg/mL with cold PBS was applied to the upper surface of the filters (5 µg/filter) and dried at room temperature under a hood. The filters thus prepared were designated Matrigel/fibronectin-coated filters. The following procedures were the same as those for the haptotactic migration assay.

Immunoblot Analysis

Western blotting was carried out according to the method described by Towbin, Staehelin *et al.* (1979) with some modifications. Briefly, 30 µL of the TCM sample was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, transferred to a

nitrocellulose membrane and immunoblotted with anti-human MMP-2 (gelatinase A) mouse monoclonal antibody at 1:1000 dilution. The biotinylated secondary antibody was used at 1:500 dilution. Immunoreactive proteins were detected with avidin-biotin-peroxidase complex (Vecstain). In negative control experiments, the primary antibody was replaced with normal mouse IgG_1.

Aminopeptidase Assay

Aminopeptidase activities were assayed by measuring the amount of 7-amino-4-methylcoumarin (AMC) liberated from amino acid-MCA by cell-associated aminopeptidases as previously described (Fujii, Nakajima *et al.*, 1995). A mixture containing 100 μM Ala-MCA or Arg-MCA and 3-4.5 x 10^4 tumor cells in 200 μL of Hank's buffer solution with or without various amounts of bestatin was placed in each well of a 96-well microplate and incubated for 90 min at 37°C. Every 30 min, the incubation mixture was mixed with 50 μL of 100 mM EDTA to terminate the reaction and the AMC level was determined with a Baxter Fluorescence Concentration Analyzer (excitation, 365 nm; emission, 450 nm. Baxter, Mandolin, IL). The activity was calculated from the amount of AMC formed by the added tumor cells.

Statistical Analysis

The significance of differences between groups was calculated by applying student's two-tailed *t*-test.

Results

Effects of Bestatin on Tumor Cell Proliferation

The effects of bestatin on the proliferation of uterine cervical carcinoma cells were studied by MTT assay. As shown in Figure 1, bestatin at the concentration of 0.1-100 μg/mL had no inhibitory or stimulatory effect on the proliferative activity of tumor cells.

Effects of Bestatin on Tumor Cell Migration

Invasion is a complex process involving cell adhesion, motility and the secretion of different classes of enzymes. Since the invasive activity of tumor cells in *in vitro* culture systems is considered to be the combined effects of tumor cell motility and enzymic degradation of ECM components, we first examined the effects of bestatin on tumor

TABLE I

Effects of Bestatin on Aminopeptidase Activities of Uterine Cervical Carcinoma Cells

Treatments (μg/mL)	Aminopeptidase Activity			
	OMC-1 (pmol/min/4.5 x 10^4 cells)		SKG-IIIb (pmol/min/3.5 x 10^4 cells)	
	Ala-MCA	Arg-MCA	Ala-MCA	Arg-MCA
Control	30.9	11.0	29.3	10.8
0.1	19.6	2.73	18.7	3.7
1	12.2	1.33	16.5	2.6
10	5.10	1.53	10.4	0.34
100	3.87	1.17	9.5	0.24
	OMC-4 (pmol/min/3.0 x 10^4 cells)		CAC-1 (pmol/min/3.0 x 10^4 cells)	
	Ala-MCA	Arg-MCA	Ala-MCA	Arg-MCA
Control	108.6	14.4	30.8	5.47
0.1	67.2	5.10	19.6	1.96
1	31.8	3.03	12.2	1.20
10	22.0	2.10	5.10	0.90
100	18.8	1.57	3.87	0.73

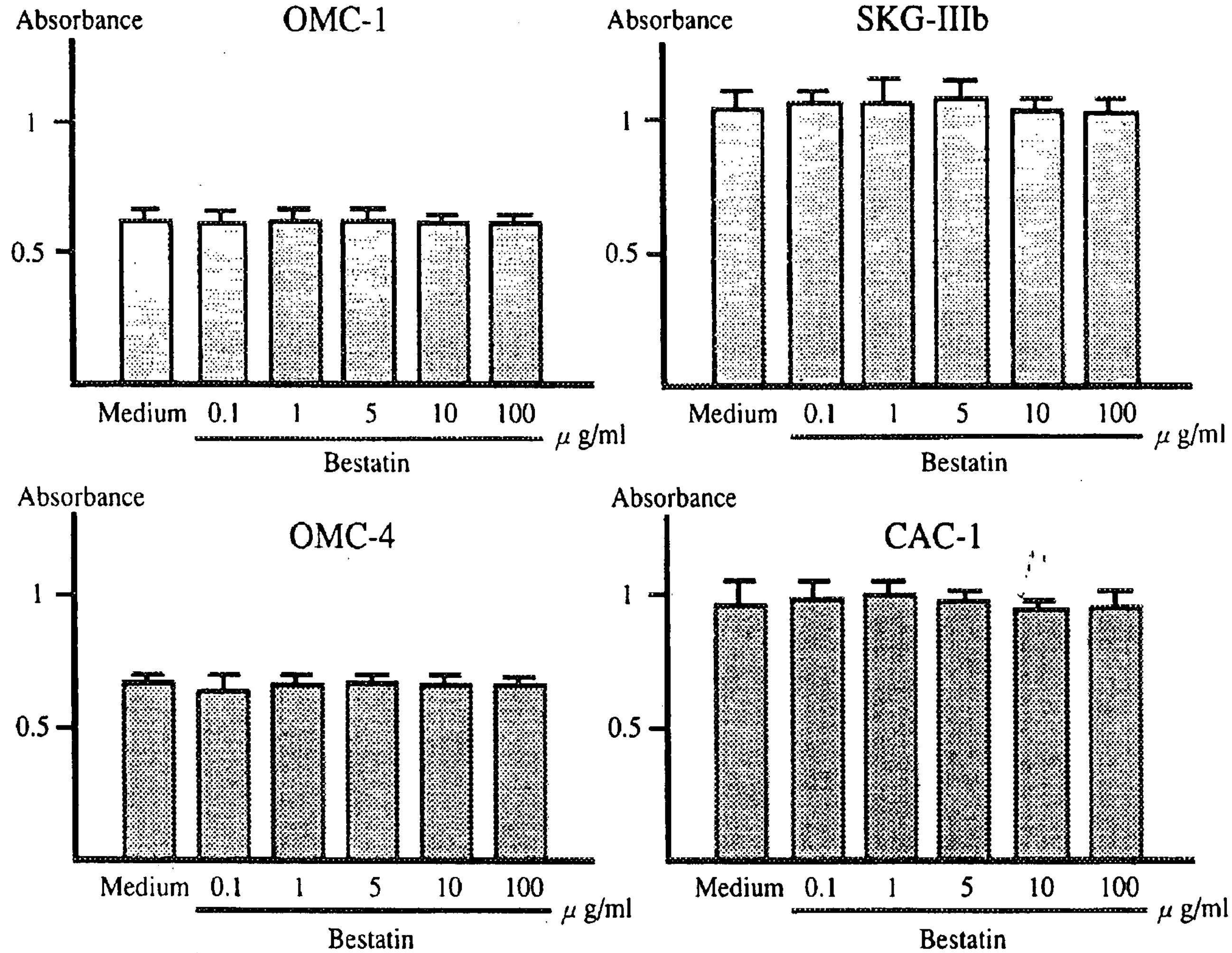

Figure 1.: Effects of bestatin on the proliferation of uterine cervical carcinoma cells. 1 x 10^4 cells were uniformly seeded into 96-well microplates and incubated for 48 hr. Each well was washed twice with serum free Ham's F-12 with 0.1% BSA and cultured for another 24 hr in the same medium. Then, 0.1-100 μg/mL of bestatin was added and each cell viability was evaluated by MTT assay after a 24 hr incubation. Bars, SD.

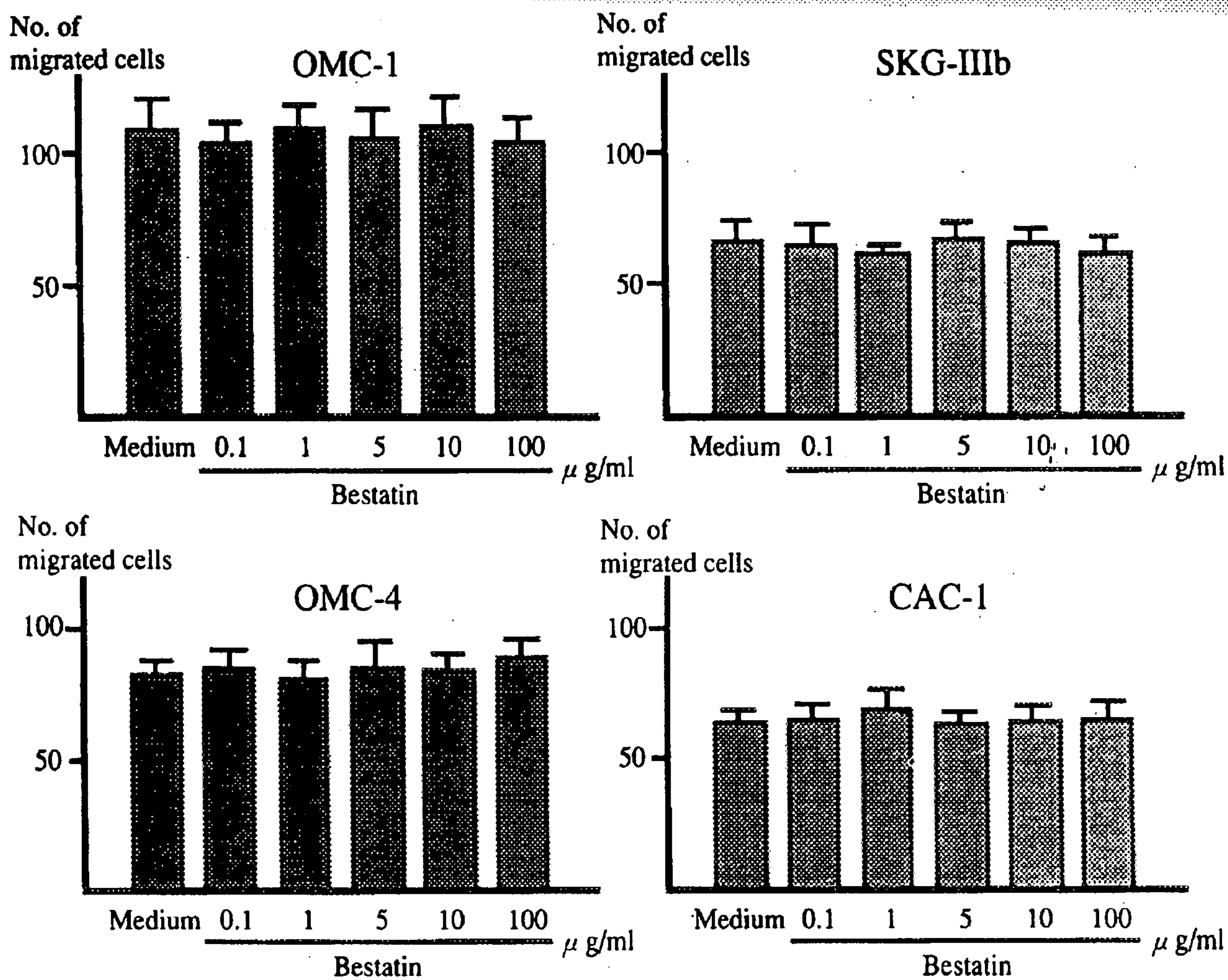

Figure 2.: Effects of bestatin on the haptotactic migration of uterine cervical carcinoma cells. 6 x 10^5 cells in Ham's F-12 with 0.1% BSA were seeded with or without 0.1-100 μg/mL of bestatin into the upper compartment of the Chemotaxicell culture chambers. Filters in chambers were precoated with 10μg of fibronectin on the lower surface. The migrated cells on the lower surface were counted under a microscope after a 24 hr incubation. Bars, SD.

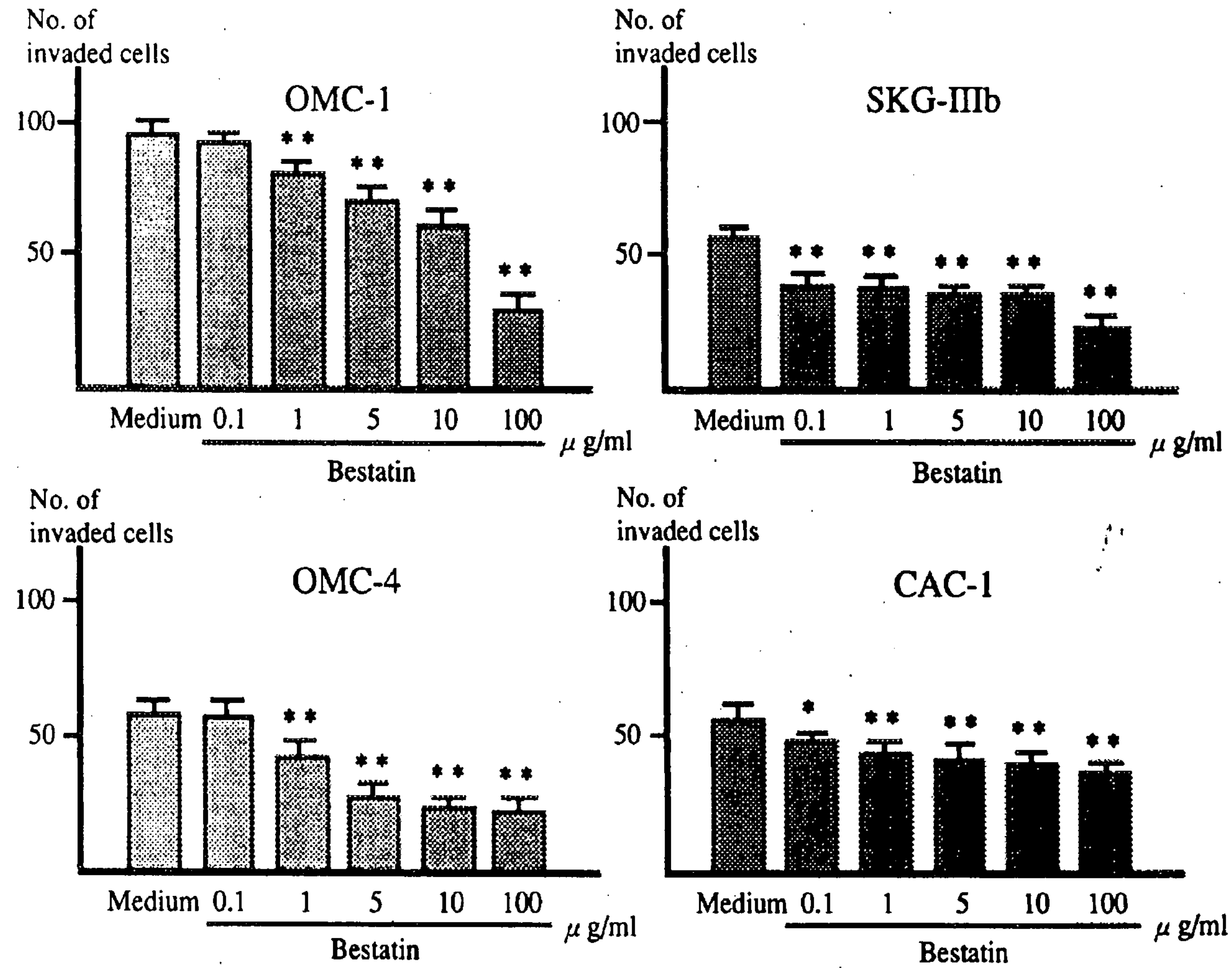

Figure 3.: Effects of bestatin on the invasive activity of uterine cervical carcinoma cells into Matrigel/fibronectin-coated filters. 6 x 10^5 cells in Ham's F-12 with 0.1% BSA were seeded with or without 0.1-100 μg/mL of bestatin into the upper compartment of the Chemotaxicell culture chambers. Filters in chambers were precoated with 10 μg of fibronectin on the lower surface, and with 5 μg of Matrigel on the upper surface. The invaded cells on the lower surface were counted under a microscope after 24 hr incubation. Bars, SD. *$p < 0.05$, **$p < 0.01$ versus control.

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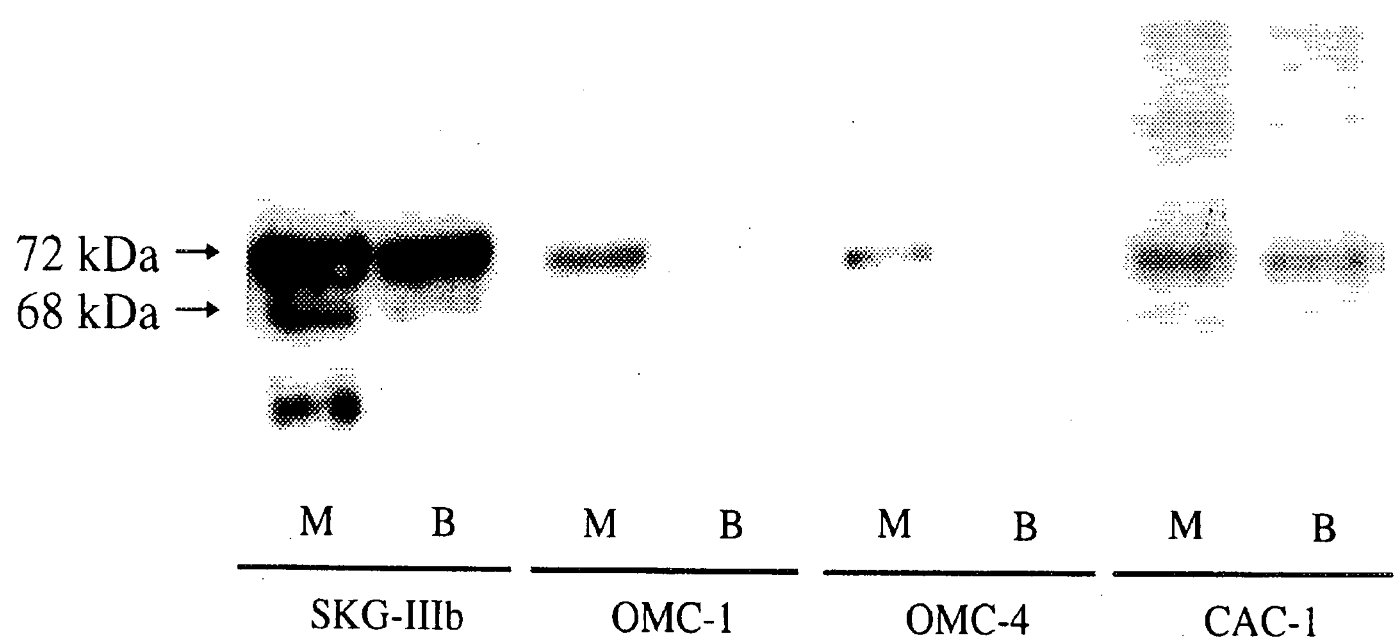

Figure 4.: Immunoblot analysis of MMP-2 (gelatinase A) in uterine cervical carcinoma cells. 30 μL of TCM samples was separated by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and immunoblotted with anti-human gelatinase A mouse monoclonal antibody at 1:1000 dilution. Immunoreactive proteins were detected with avidin-biotin-peroxidase complex. M: Medium, B: Bestatin 100 μg/mL.

cell migration. As shown in Figure 2, haptotactic migration of uterine cervical carcinoma cells along a gradient of substratum-bound fibronection was not inhibited or stimulated by 0.1-100 μg/mL of bestatin.

Effects of Bestatin on Tumor Cell Invasion

Uterine cervical carcinoma cells were incubated for 24 hr at 37°C with bestatin at the concentration of 0.1-100 μg/mL in the upper compartment of the Chemotaxicell culture chambers. As shown in Figure 3, the invasive activity of tumor cells into Matrigel/fibronectin-coated filters was inhibited by the presence of bestatin in a concentration-dependent manner. Among four cell lines examined, lower concentrations of bestatin (0.1-1 μg/mL) drastically inhibited the invasive activity of SKG-IIIb cells, which were considered to be most sensitive for bestatin in *in vitro* invasion assays.

Effects of Bestatin on the Expression of Gelatinase A in Immunoblot Analysis

To further investigate the effects of bestatin on the invasion process of uterine cervical carcinoma cells, we next performed immunoblot analysis for TCM using anti-human MMP-2 (gelatinase A) monoclonal antibody. As shown in Figure 4, the treatment of tumor cells with bestatin inibited the expression of the 72 kDa gelatinase level (gelatinase A, latent form) in the four cell lines examined, and the reduction of the 68 kDa gelatinase level (gelatinase A, active form) was also found in SKG-IIIb cells. In SKG-IIIb cells, the other band with a molecular weight of 50 kDa was detected, and its expression was inhibited by bestatin. This band might be a degradation product of gelatinase A without enzymatic activities.

Effects of Bestatin on Aminopeptidase Activities in Tumor Cells

We also examined whether or not bestatin could inhibit the aminopeptidase activities in uterine cervical carcinoma cells by measuring the amount of AMC liberated from amino acid-MCA. As shown in Table I, bestatin at the concentration of 0.1-100 μg/mL inhibited the Ala-MCA- and Arg-MCA- hydrolyzing activities of the four cell lines examined in a concentration-dependent manner. The direct effect of bestatin on gelatinase A was also examined by

zymography with gelatin as a substrate. Even in the presence of 100 μg/mL bestatin through the preincubation and incubation periods, gelatinase A activity was not significantly affected (data not shown).

Discussion

In this study, bestatin inhibited the invasion of OMC-1, SKG-IIIb, OMC-4 and CAC-1 cells into the reconstituted basement membrane Matrigel in a concentration-dependent manner. The penetration of tumor cells into basement membranes involves distinct events including cell attachment, degradation of the adjacent ECM components caused by proteolytic enzymes secreted by tumor cells, and migration of the cells into the target tissue. The invasive activity of tumor cells in *in vitro* culture systems is considered to be the combined effects of tumor cell motility and enzymatic degradation of Matrigel components. However, bestatin did not have any effect on the motility of cultured uterine cervical carcinoma cells to fibronectin substrates, nor did it affect the growth of these cells in our experimental conditions. Therefore, it is likely that the inhibitory effect of bestatin on the invasive activity of cervical carcinoma cells is associated with the degradative cascade of ECM.

Liotta, Tryggvason *et al.* (1980) and Nakajima, Welch *et al.* (1987) have shown that there are substantial correlations between type IV collagenolytic activities and the invasive and metastatic potentials for a variety of human and animal tumor cell lines. Immunoblot analysis of TCM showed that the treatment of tumor cells with bestatin resulted in the reduction of the 72 kDa gelatinase level, and the reduction of the 68 kDa gelatinase level was also found in SKG-IIIb cells. It is well known that gelatinases exist in both latent and active forms (Woessner, 1991). Two bands were shown to be the active (68 kDa) and the latent (72 kDa) forms of gelatinase A. Bestatin may inhibit the conversion of the latent form of gelatinase A to the active form in SKG-IIIb cells. Yoneda, Saiki *et al.* (1992) also reported a similar effect of bestatin in SN12M cells, suggesting that plasma membrane aminopeptidases may be involved in the activation mechanism of gelatinases as well as the other matrix proteinases such as plasmin or stromelysin.

Several authors (Fujii, Nakajima *et al.*, 1995; Saiki, Fujii *et al.*, 1993; Saiki, Murata *et al.*, 1989; Yoneda, Saiki *et al.*, 1992) have previously demonstrated that bestatin, a potent inhibitor of

aminopeptidases, suppresses the degradation and invasion of ECM by tumor cells. Saiki, Fujii *et al.* (1993) also reported that the anti-aminopeptidase N/CD 13 monoclonal antibody, capable of inhibiting aminopeptidase, can suppress the invasive activity of tumor cells. These findings have suggested the possible involvement of aminopeptidase N/CD 13 in the molecular mechanisms of tumor cell invasion.

We then examined the effects of bestatin on aminopeptidase activities in uterine cervical carcinoma cells by measuring AMC liberated from amino acid-MCA. Bestatin inhibited hydrolyzing activities towards Ala-MCA and Arg-MCA in these cells. The present results suggest that bestatin may inhibit the invasive activity of uterine cervical carcinoma cells through a mechanism involving its inhibitory action on aminopeptidases in the cells. Yoneda, Saiki *et al.* (1992) showed that inhibitory action of bestatin on aminopeptidases in tumor cells was partly associated with the conversion of the latent form of type IV collagenase to the active form or the secretion of collagenases from tumor cells. In our results, bestatin induced a slight reduction of the 72 kDa gelatinase level in four cell lines, and a significant reduction of the 68 kDa gelatinase level in SKG-IIIb cells. This may be related to its inhibitory action on aminopeptidase activities of the cells and subsequent effects on the secretion of gelatinase A and the conversion of the latent form of gelatinase A to the active form. Moreover, there was no direct inhibition of gelatinase A activity by bestatin.

These results suggest that bestatin, an aminopeptidase inhibitor, potently inhibits the invasive activity of uterine cervical carcinoma cells, possibly through the inhibitory mechanism for production and activation of gelatinase A modulated by tumor aminopeptidases.

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